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13. ABSTRACT (Maximum 200 Words)

Elevated serum levels of insulin-like growth factor 1 (IGF1) have been found in prostate cancer patients, and IGF1-related signal transduction is thought to be an important factor in the development of prostate cancers. The goals of this project are to discover small organic molecules that suppress IGF-activated prostate cancers by cell-based screening and to analyze their action mechanisms. We have been taking a unique two-step approach to discovering such molecules: we first examine the phenotypic effects of chemical library members (10,000 divergent drug-like compounds) on the insulin-induced adipogensis and, by using the adipogenesis profile, we then identify organic compounds that suppress IGF-mediated growth of prostate cancer cells. Our hypothesis is that some of the compounds that block the insulin-induced adipogenesis suppress IGF-stimulated proliferation of malignant prostate cancers. In the first year of funding, we discovered, from the pool of chemicals that blocked insulin-induced adipogenesis, the drug-like compound we call 125B11 that suppress IGF1-dependent growth of prostate cancer cells but not serum-dependent growth. We are currently working on its mechanisms of action.

14. SUBJECT TERMS

Insulin-like growth factor, small molecule, chemical library, Chemical genetics, DNA microarray

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Table of Contents

Introduction

Elevated serum levels of insulin-like growth factor 1 (IGF1) have been found in prostate cancer patients, and IGF1-related signal transduction is thought to be an important factor in the development of prostate cancers (*1*). The goals of this project are to discover small organic molecules that suppress IGF-activated prostate cancers by cell-based screening and to analyze their action mechanisms. We have been taking a unique two-step approach to discovering such molecules: we first examine the phenotypic effects of chemical library members (10,000 divergent drug-like compounds) on the insulin-induced adipogenic differentiation of cultured fibroblasts and, by using the adipogenesis profile, we then identify organic compounds that suppress IGF-mediated growth of prostate cancer cells. Our hypothesis is that some of the compounds that block the insulin-induced adipogenesis may suppress IGF-stimulated proliferation of malignant prostate cancers. The compounds may serve as a seed for a new type of anticancer drugs that could treat prostate cancers and also as a tool to understand the IGF signaling in malignant prostate cancers.

Body

In the first year of funding, we focused on Task 1:
To isolate small molecules that specifically inhibits the IGF-induced cell growth of prostate cancer cells

Adipogenesis profiling for discovering anticancer agents (*2*). Our approach to discovering anticancer agents is based on the logic of genetics. In genetic screens, clear morphological phenotypes are often used just as a sensitive tool for discovering and analyzing genes whose primary functions are seemingly unrelated to the morphological phenotype. A good example is the use of eye morphology in the fruit fly *Drosophila melanogaster* as a genetic tool for the analysis of genes in disease-linked signaling pathways (*3*). Although human diseases associated with these pathways, such as cancer and neurodegenerative diseases, are seemingly unrelated to eye development, the use of eye morphology as a sensitive indicator enabled a systematic understanding of the disease-linked signaling events (*4-8*). We envisioned that clear morphological phenotypes of cells could similarly be used as a sensitive indicator of the drug effects that are not associated directly with the morphological phenotypes.

The morphological alteration we used is the differentiation of murine 3T3-L1 fibroblasts into adipocytes, one of the most drastic and sensitive morphological alterations in cultured mammalian cells (*9*). In the presence of insulin, 3T3-L1 cells undergo differentiation into adipocytes, which are visually distinct from the original cells because of the presence of oil droplets in the cytoplasm. The insulin-induced adipogenesis of 3T3-L1 cells involves a number of disease-linked proteins such as PI3K, Ras, PPARγ, p38, or phosphodiesterases, and known drugs for a range of diseases have been reported to have phenotypic effects on the adipogenesis

(*9-14*). A morphology-based adipogenesis screen of a chemical library could identify a pool of biologically active compounds with many distinct pharmacological effects including anticancer ones.

Adipogenesis profile of 10,000 divergent drug-like compounds (*2*). The divergent chemical library used for our case study was a Prime-Collection 2000 Format Q (ChemBridge). In this format, 10,000 drug-like molecules are rationally preselected to form a library that covers the maximum pharmacore diversity with the minimum number of compounds. Two academic groups have reported successful isolations of unique compounds from a similar chemical library (*15, 16*), indicating that this type of chemical libraries contains a diverse set of compounds that are suited for a proof-of-principle study. Our cell-morphology profiling of the 10,000-compound library identified 188 chemicals that clearly modulated the insulin-induced differentiation of 3T3-L1 cells at 20 ng/μL (**Fig. 1**): eighty-one compounds potentiated the adipogenesis, eighty-seven compounds completely blocked the differentiation, and thirteen compounds induced other morphological phenotypes such as adipocyte-like cells without oil droplets. The screen thus reduced pool of chemicals by 53 fold. The adipogenesis-modulating activity of selected compounds were confirmed by RT-PCR analysis of

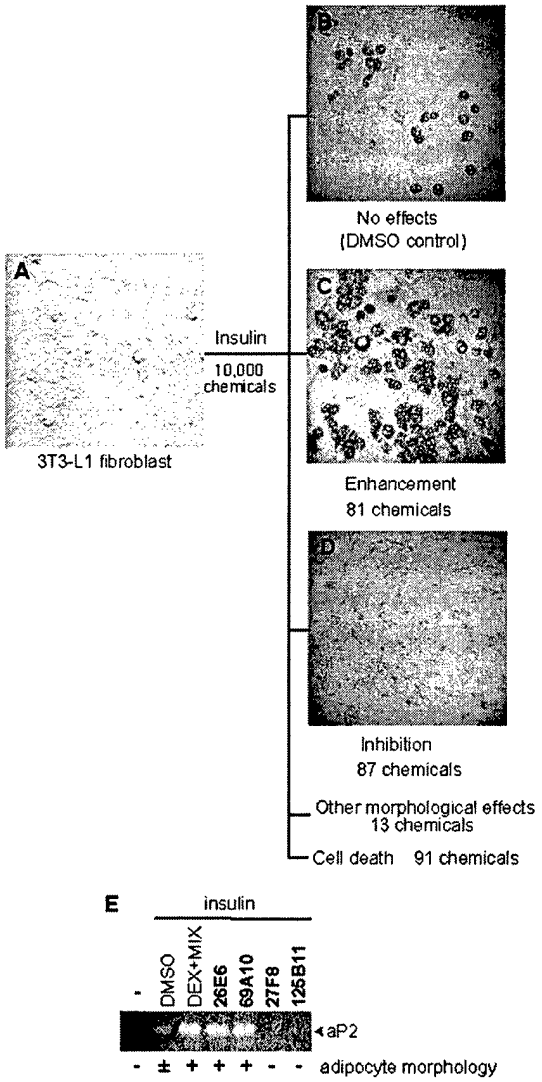

Fig. 1. Adipogenesis profiling of a library of 10,000 divergent drug-like compounds. 3T3-L1 cells have a morphology characteristic of fibroblasts (**A**). After chemical treatment in the presence of insulin, the cell morphology was examined under microscope. The control wells that are treated with 1% (v/v) DMSO have about 5% adipocytes (**B**). The compounds that enhanced adipogenesis more than five folds were scored to be adipogenesis-enhancing chemicals (**C**), and the compounds that completely inhibited adipogenesis without detectable cytotoxicity were scored to be adipogenesis-blocking chemicals (**D**). (**E**) RT-PCR analysis of adipocyte-specific aP2. 3T3-L1 cells were treated with chemicals for three days, and total RNA was isolated at day seven. Typical results of four representative compounds are shown along with the positive control of 1 μM dexamethasone (DEX) and 0.5 mM methylisobutylxanthine (MIX).

aP2, an adipocyte-specific fatty acid-binding protein (an example is shown in **Fig. 1E**). The 188 adipogenesis-modulating chemicals that we found are apparently non-toxic for confluent 3T3-L1 cells and almost certainly modulate particular biologic responses in mammalian cells. In other words, the collection of 188 chemicals is a biology-focused chemical library.

Suppressors of IGF-activated prostate cancer cells (*2*). Both insulin and IGFs stimulate oncogenic signaling pathways including those of Ras-MAPK and PI3K-Akt, and overexpression of IGFs is often associated with cancer malignancy (*17*). Patients with IGF-overexpressing tumors tend to have severe hypoglycemia despite low levels of serum insulin (known as non-islet cell tumor hypoglycemia) (*18*), demonstrating a functional overlap between oncogenic IGFs and insulin in vivo. These considerations led to the hypothesis that the pool of the adipogenesis-blocking chemicals contains anticancer compounds that suppress the IGF-stimulated survival and proliferation of malignant tumor cells. For a chemical screen, we used DU-145 androgen-independent prostate cancer cells whose growth can be stimulated by IGF1 as much as by 2% serum. The pool of the 87 adipogenesis-blocking chemicals contained two analogous chemicals that specifically inhibited the IGF1-induced growth of DU-145 cells but had little effects on their serum-induced growth. One of them, 125B11, had the greatest differential activity; the simple drug-like thiazole derivative impaired the IGF1-induced growth at an IC_{50} of 0.1 μM but had little effects on the serum-dependent growth (**Fig. 2**). IGF1-induced phosphorylation of Akt and MAPK in DU-145 cells was unaffected by 125B11, suggesting that 125B11 inhibits the cell-proliferative function of IGF1 in a way independent of the known IGF1-signaling pathway. We are currently working on the mechanisms of action of 125B11 through biochemical and DNA microarray analyses. We are also obtaining structure-activity relationship of 125B11.

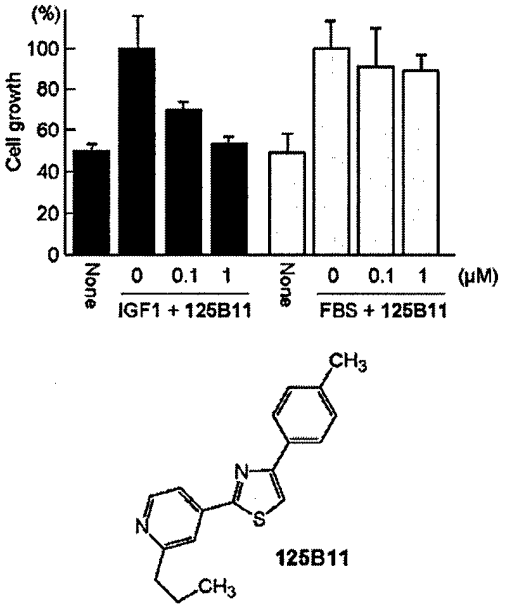

Fig. 2. Discovery of 125B11. 125B11 inhibited the IGF1-induced growth but not the serum-induced growth. DU-145 cells were treated with varied amounts of 125B11 in the presence of IGF1 or 2% fetal bovine serum (FBS).

Key research accomplishments

- Obtained experimental results supporting our hypothesis that adipogenesis profiling of organic molecules is useful for discovering IGF suppressing compounds
- Discovered the unique compound 125B11

- Obtained evidence suggesting that 125B11 has an unprecedented mechanism of action.

Reportable outcomes

- Identification of bioactive molecules by adipogenesis profiling of organic compounds Choi, Y., Kawazoe, Y., Murakami, K., Misawa, H., **Uesugi, M.** *J. Biol. Chem.* 278, 7320-7324 (2003).
- Identification of bioactive molecules by adipogenesis profiling of organic compounds Choi, Y., Kawazoe, Y., Murakami, K., **Uesugi, M.** *FASEB Journal,* 17 (4): A605-A605 (2003).

Conclusion

Fat cell differentiation *per se* has no direct link to suppression of IGF1-activated prostate cancer cells. Nevertheless, our proof-of-principle study using a 10,000-compound library successfully identified a non-cytotoxic IGF1-suppressing compound, just as genetics has identified non-lethal disease-linked genes by examining the eye morphology of fruit flies.

One potential drawback of our approach is that the bioactive molecules from the adipogenesis-based focused library may have side effects that are associated with adipogenesis. However, some degree of side effects are usually expected for any unoptimized molecules, and classical medicinal chemistry approaches have been taken for reducing the unwanted side effects. The high sensitivity of the morphological transformation of 3T3-L1 cells also suggests that the adipogenesis-modulating effects of chemicals may not necessarily be reproduced in human. For instance, non-steroidal anti-inflammatory drugs and phosphodiesterase inhibitors such as aspirin and caffeine are known to enhance adipogenesis of 3T3-L1 cells, but have no significant effects on fat accumulation in human. The adipogenesis profiling is perhaps a good filter for lead-like IGF suppressors that can be used for further biological, chemical genetic, and medicinal chemical studies.

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Appendix

- One reprint copy of the paper that we published in *J. Biol. Chem.*

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Identification of Bioactive Molecules by Adipogenesis Profiling of Organic Compounds*[S]

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An important step in the postgenomic drug discovery is the construction of high quality chemical libraries that generate bioactive molecules at high rates. Here we report a cell-based approach to composing a focused library of biologically active compounds. A collection of bioactive non-cytotoxic chemicals was identified from a divergent library through the effects on the insulin-induced adipogenesis of 3T3-L1 cells, one of the most drastic and sensitive morphological alterations in cultured mammalian cells. The resulting focused library amply contained unique compounds with a broad range of pharmacological effects, including glucose-uptake enhancement, cytokine inhibition, osteogenesis stimulation, and selective suppression of cancer cells. Adipogenesis profiling of organic compounds generates a focused chemical library for multiple biological effects that are seemingly unrelated to adipogenesis, just as genetic screens with the morphology of fly eyes identify oncogenes and neurodegenerative genes.

A complete analysis of human genome is anticipated to produce an unprecedented number of potential drug targets. The development of high throughput assays for these genomic pseudotargets may be a challenging but important step for not limiting drug discovery to the "relatively easy" targets such as G-protein-coupled receptors or particular enzymes. An alternative or complementary effort is the construction of high quality chemical libraries that generate bioactive molecules at higher rates. The small size of the focused libraries would lower the cost of screening processes and enable unique low throughput screens, extending the scope of assays for the genomic targets and for a given therapeutic effect.

Our approach to constructing a focused chemical library is based on the logic of genetics. In genetic screens, clear morphological phenotypes are often used just as a sensitive tool for discovering and analyzing genes whose primary functions are seemingly unrelated to the morphological phenotype. A good example is the use of eye morphology in the fruit fly *Drosophila melanogaster* as a genetic tool for the analysis of genes in disease-linked signaling pathways (1). Although human diseases associated with these pathways, such as cancer and neurodegenerative diseases, are seemingly unrelated to eye development, the use of eye morphology as a sensitive indicator enabled a systematic understanding of the disease-linked signaling events (2–6). We envisioned that clear morphological phenotypes of cells could similarly be used as a sensitive indicator of the drug effects that are not associated directly with the morphological phenotypes.

The morphological alteration we used is the differentiation of murine 3T3-L1 fibroblasts into adipocytes, one of the most drastic and sensitive morphological alterations in cultured mammalian cells (7). In the presence of insulin, 3T3-L1 cells undergo differentiation into adipocytes, which are visually distinct from the original cells because of the presence of oil droplets in the cytoplasm (Fig. 1). The insulin-induced adipogenesis of 3T3-L1 cells involves a number of disease-linked proteins such as phosphatidylinositol 3-kinase, Ras, peroxisome proliferator-activated receptor γ, p38, or phosphodiesterases, and known drugs for a range of diseases have been reported to have phenotypic effects on the adipogenesis (7–12). A morphology-based adipogenesis screen of a chemical library could identify a pool of biologically active compounds with many distinct pharmacological effects. Here we report a proof-of-principle study using a library of 10,000 divergent compounds.

EXPERIMENTAL PROCEDURES

Adipogenesis Profiling—3T3-L1 fibroblasts were plated in 96-well plates at a density of 5×10^4 cells/well and allowed to reach maximal confluence. The confluent cells were treated individually with 20 ng/μl of a chemical for 3 days in 100 μl of Dulbecco's modified Eagle's medium containing of insulin (5 μg/ml) and 10% fetal bovine serum (FBS).[1] After the removal of insulin and the chemical, the cells were further maintained typically for 8 days with the replacement of media every 3 days. The effects of chemicals on the adipogenesis were evaluated under microscope. The control wells with 1% (v/v) Me_2SO had ~5% adipocytes. The compounds that enhanced the adipogenesis >5-folds were scored to be adipogenesis-enhancing chemicals, and the ones that completely inhibited adipogenesis without detectable toxicity were scored to be adipogenesis-blocking chemicals. The effects of these chemicals were confirmed multiple times by multiple laboratory members. Cell viability was monitored by trypan blue exclusion and by counting cell numbers.

Reverse Transcription (RT)-PCR—Total RNA was isolated with TRI-reagent (Molecular Research Center) at day 7 (aP2) or day 3 (osteocalcin). 5 μg of total RNA was reverse-transcribed to cDNA by using oligo(dT) primer with avian myeloblastosis virus reverse transcriptase for 60 min at 42 °C. The cDNA was then amplified by using Ex*Taq*

* This work was supported in part by U. S. Department of Defense Prostate Cancer Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. 1 and 2.

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[1] The abbreviations used are: FBS, fetal bovine serum; RT, reverse transcription; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; IGF, insulin-like growth factor; SEAP, secreted alkaline phosphatase; AP, activating protein; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

(Takara) with following primer pairs: 5′-AACACCGAGATTTCCTTCAA-3′ and 5′-TCACGCCTTTCATAACACAT-3′ for aP2; 5′-TCTGACAAACCTTCATGTCC-3′ and 5′-AAATAGTGATACCGTAGATGCG-3′ for osteocalcin. The amplification conditions are as follows: 95 °C (30 s), 60 °C (30 s), 72 °C (30 s) for 23 cycles (aP2) or 30 cycles (osteocalcin).

Glucose Uptake Study—3T3-L1 fibroblasts were induced to differentiate into adipocytes by incubation in a medium containing 10% FBS, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, and 1.7 μM insulin. After 2 days, the medium was switched to the one containing 10% FBS and 1.7 μM insulin for 2 days and then to a normal 10% FBS medium for 3 days. After the total of 7 days, almost 100% 3T3-L1 cells were differentiated into adipocytes. These fully differentiated cells were treated on 24-well plates with varied concentrations of chemicals (0.1% Me_2SO) for 24 h and then incubated with 100 nM insulin and 2-[^{3}H]deoxyglucose. The cells were extensively washed, and their radioactivity was measured by scintillation counting. All of the samples were tested in duplicate.

Cytokine Production Assay—For the analysis of IL-6 and TNF-α, the mouse macrophage cell line RAW264.7 was used. Cells were seeded onto 96-well plates, and the cytokine production was induced by adding 10 μg/ml lipopolysaccharide. Upon stimulation, each one of the adipogenesis-enhancing chemicals was also added to the culture at varied concentrations. After incubating for 48 h at 37 °C, the cytokine concentrations in the culture supernatants were measured by ELISA. For the analysis of IL-2, the mouse thymoma cell line EL-4 was used, and the IL-2 production was induced by adding phorbol ester and ionophore. The effects of chemicals on the IL-2 production were similarly examined by ELISA. All of the samples were tested in triplicate.

Mineralization Assay—The clonal osteoblastic cell line MC3T3-E1, clone 14, was grown in α-minimum Eagle's medium supplemented with 10% FBS until confluent in 96-well plates. For induction of mineralization, the cells were further incubated with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate in the presence or absence of chemicals. On day 14, the cells were washed with phosphate-buffered saline, fixed in 10% formalin, and washed with distilled water. Bonelike mineral formation was evaluated by examining the area stained by 2% (w/v) Alizarin Red S (pH 4.2).

Assays for Insulin-like Growth Factor (IGF)-activated Cancer Cells—The adipogenesis-blocking chemicals were assayed for their ability to inhibit the growth of IGF-activated cancer cells. For the discovery of inhibitors of IGF2, we used five distinct human hepatocellular carcinoma cell lines, Hep-G2, SK-Hep-1, and three lines that we recently characterized.[2] Three of them produce IGF2 at high levels, whereas two express ~10 times less amounts of IGF2 as measured by ELISA, RT-PCR, and DNA microarray experiments. Treatment with a neutralizing antibody against IGF2 selectively inhibited the growth of the IGF2-overexpressing cell lines but had little effects on that of the cell lines with low levels of IGF2. Thus, these cell lines served as an excellent system for discovering the chemicals that selectively impair the growth of IGF2-overexpressing hepatocellular carcinoma cells. For cell viability assays, IGF2-expressing cells were plated at a density of 4×10^3 onto 96-well plates. After a 24-h incubation, the cells were treated with varied amounts of chemicals for 72 h. The effects of chemicals were evaluated by microscopic observation and MTT assay. All of the samples were tested at least three times. For reporter gene assays, IGF2-expressing cells were transfected with a reporter construct in which a gene encoding secreted alkaline phosphatase (SEAP) is controlled by the IGF2 promoter, AP-1 sites, NFkB sites, or the SV40 promoter. After 24 h, the transfected cells were treated with 94G6 (0.1 μM) for 8 h. SEAP activity was measured through fluorescence change of methylumbelliferyl phosphate. The experiments were repeated six times. For the discovery of inhibitors of IGF1, we used DU-145, a human androgen-independent prostate cancer cell whose growth can be stimulated by IGF1 in a non-serum medium (13). Chemicals that inhibit the IGF1-induced growth of DU-145 but not its serum-dependent growth were searched in the focused library of adipogenesis-blocking chemicals. DU-145 cells were seeded onto 96-well plates at a density of 2,000 cells/well in the presence of 1 μg/ml IGF1 or 2% FBS. After 24 h, chemicals were added to the culture at varied concentrations. Cell proliferation was estimated by MTT assays after 3 days. The experiments were performed in triplicate.

RESULTS

Adipogenesis Profiling of 10,000 Divergent Compounds—The divergent chemical library used for our case study was a Prime Collection 2000 Format Q (ChemBridge). In this format, 10,000 druglike molecules are rationally preselected to form a library that covers the maximum pharmacore diversity with the minimum number of compounds. Two academic groups (14, 15) have reported successful isolations of unique compounds from a similar chemical library, indicating that this type of chemical library contains a diverse set of compounds that are suited for a proof-of-principle study. Our cell morphology profiling of the 10,000-compound library identified 188 chemicals that clearly modulated the insulin-induced differentiation of 3T3-L1 cells at 20 ng/μl (Fig. 1): 81 compounds potentiated the adipogenesis; 87 compounds completely blocked the differentiation; and

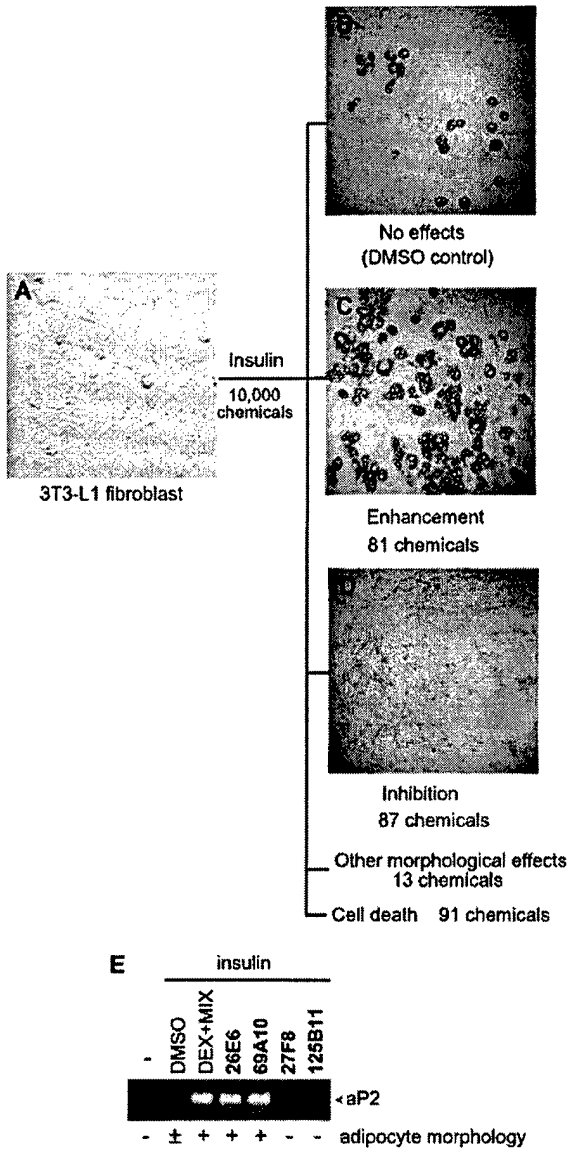

FIG. 1. **Adipogenesis profiling of a library of 10,000 divergent druglike compounds.** *A*, 3T3-L1 cells have a morphology characteristic of fibroblasts. After chemical treatment in the presence of insulin, the cell morphology was examined under microscope. *B*, the control wells that are treated with 1% (v/v) Me_2SO (*DMSO*) have ~5% adipocytes. The compounds that enhanced adipogenesis >5-folds were scored to be adipogenesis-enhancing chemicals (*C*), and the compounds that completely inhibited adipogenesis without detectable cytotoxicity were scored to be adipogenesis-blocking chemicals (*D*). *E*, RT-PCR analysis of adipocyte-specific aP2. 3T3-L1 cells were treated with chemicals for 3 days, and total RNA was isolated at day 7. Typical results of four representative compounds are shown along with the positive control of 1 μM dexamethasone (*DEX*) and 0.5 mM methylisobutylxanthine (*MIX*).

[2] K. Murakami and M. Uesugi, unpublished data.

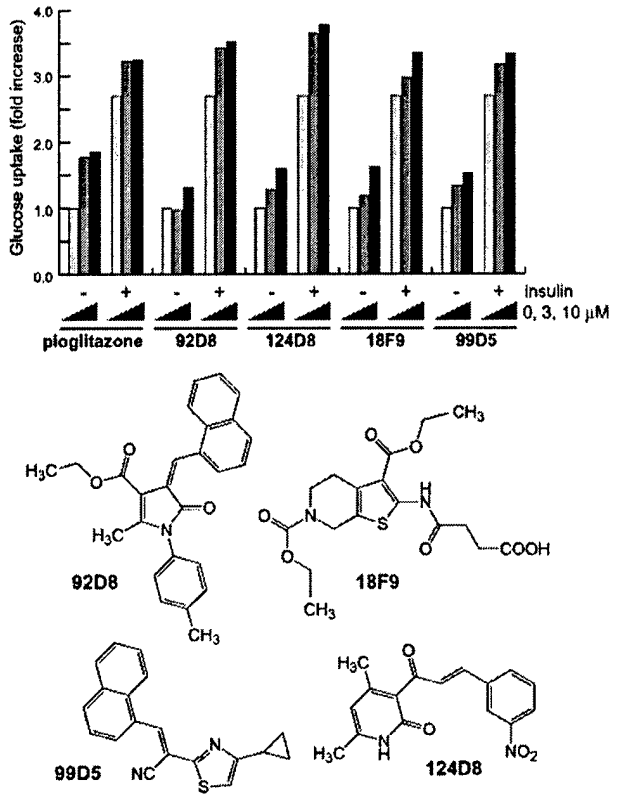

FIG. 2. **Identification of glucose-uptake enhancers.** Fully differentiated adipocytes were treated with 3 or 10 μM chemicals and 2-[^{3}H]deoxyglucose in the presence or absence of 100 nM insulin on 24-well plates. Glucose uptake was measured by scintillation counting. The results of the best four chemicals are shown.

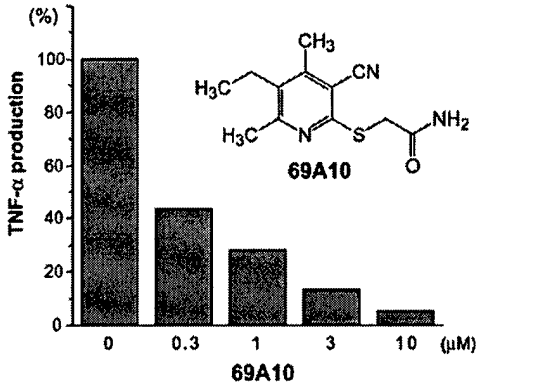

FIG. 3. **Inhibition of TNF-α production by 69A10.** Macrophage RAW264.7 cells were seeded onto 96-well plates, and TNF-α was induced by adding lipopolysaccharide. Upon the stimulation, 69A10 was added to the culture. After incubating for 48 h, the TNF-α concentrations were measured by ELISA.

13 compounds induced other morphological phenotypes such as adipocyte-like cells without oil droplets. Thus, the screen reduced a pool of chemicals by 53-fold. The adipogenesis-modulating activity of selected compounds was confirmed by RT-PCR analysis of aP2, an adipocyte-specific fatty acid-binding protein (an example is shown in Fig. 1*E*). The 188 adipogenesis-modulating chemicals that we found are apparently non-toxic for confluent 3T3-L1 cells and almost certainly modulate particular biologic responses in mammalian cells. The chemical structures of adipogenesis-enhancing and -blocking compounds are disclosed in supplementary Figs. 1 and 2.

Glucose-uptaking Insulin Sensitizers—We first focused on the 81 chemicals that potentiated the insulin-induced adipogenesis. Their insulin-sensitizing activity in the adipocyte differentiation suggests that some of them enhance the insulin-induced glucose uptake with anti-diabetic properties. This prediction was supported by the fact that the thiazolidinedione family of anti-diabetic drugs enhances the adipogenesis of 3T3-L1 cells through the activation of peroxisome proliferator-activated receptor γ, a nuclear receptor that plays an important role in adipocyte differentiation (16). In fact, among the adipogenesis-enhancing compounds, nine had a structural element chemically equivalent to thiazolidinedione. These known chemicals were eliminated, and the remaining 72 chemicals were assayed for their ability to potentiate insulin-induced glucose uptake in cultured adipocytes. The 72 compounds contained as many as 11 molecules that enhanced the glucose uptake at comparable levels with that of pioglitazone, a clinically used anti-diabetic drug, demonstrating the validness of our approach. Four of them exhibited insulin-sensitizing activity stronger than pioglitazone at 10 μM, and the most potent one was 124D8 (Fig. 2). Its kinase-inhibitor-like structure is novel as an insulin sensitizer and appears to modulate the function of insulin independently from the major insulin pathways because 124D8 had no effects on the phosphorylation of Akt and MAPK in 3T3-L1 cells. Adipogenesis profiling of a larger chemical library is likely to generate a number of glucose-uptaking compounds with a novel mechanism of action.

Inhibitors of Inflammatory Cytokine Production—Recent studies (7, 12) suggest a cross-talk between insulin-induced adipogenesis and inflammatory responses. Anti-inflammatory drugs including glucocorticoid, phosphodiesterase inhibitors, and salicylates stimulate insulin-induced adipogenesis of 3T3-L1 cells, and molecular targets for anti-inflammatory drugs such as p38, TNF-α, and IL-1 are involved in adipogenesis or insulin resistance of somatic cells (11, 17–19). Although the molecular mechanism of the cross-talk remains unclear, these lines of evidence implicate the presence of anti-inflammatory compounds in the pool of the adipogenesis-enhancing chemicals. We assayed the 72 adipogenesis-enhancing chemicals for their ability to reduce the production of three inflammatory cytokines, IL-6, IL-2, and TNF-α. Eighteen compounds inhibited the production of a cytokine >50% at 10 μM without notable cytotoxicity, suggesting a high density of cytokine production inhibitors in the adipogenesis-enhancing chemicals. Among those, the compound that we call 69A10 inhibited the TNF-α production in macrophage RAW cells with a IC_{50} of 0.3 μM (Fig. 3). A focused library of adipogenesis-enhancing chemicals may be useful for identifying anti-TNF-α compounds, and their mechanistic studies would clarify the interesting cross-talk between adipogenesis and inflammatory responses.

Osteogenesis Stimulators—Insulin shares sequence homology and biological activity with IGFs. Deficiency in IGF1, a prominent member of IGFs, is suggested to be a cause of decrease in bone density with aging (20, 21), and administration of IGF1 prevents the decrease of bone density in osteoporosis patients in part by stimulating osteogenesis (22–24). The high homology between IGF1 and insulin suggested that the osteogenesis-enhancing activity of IGF1 may be mimicked by the chemicals that potentiated the insulin-induced adipogenesis. As a quick test, the adipogenesis-enhancing chemicals were assayed for their ability to stimulate the formation of bonelike mineral deposition in MC3T3-E1 cells. We found three compounds that increased the mineralization at 5 μM as much as IGF1 or ipriflavone, a clinically used anti-osteoporosis drug (Fig. 4*A*). Their osteogenesis-stimulating activity was confirmed by RT-PCR analysis of osteocalcin, a marker gene of osteoblastic differentiation. The three compounds exhibited an

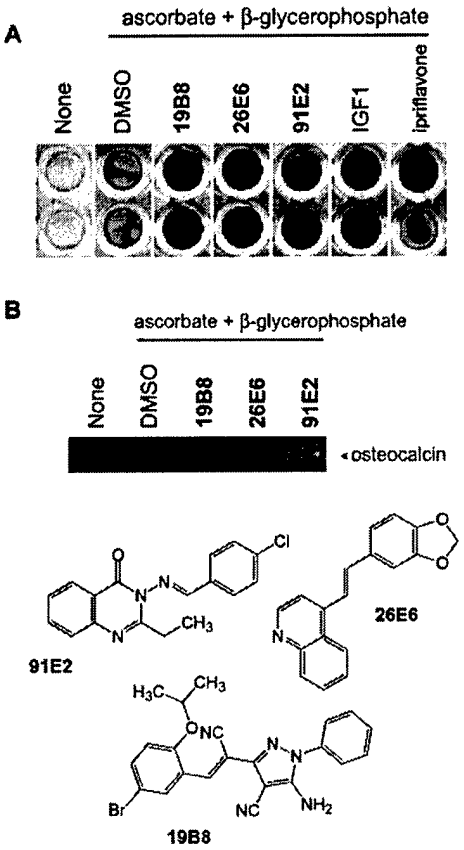

FIG. 4. **Effects of 19B8, 26E6, and 91E2 on the osteogenesis of MC3T3-E1 cells.** *A*, mineralization assay. MC3T3-E1 cells were treated with 1% (v/v) Me_2SO (*DMSO*) or 5 μM 19B8, 26E6, or 91E2 for 14 days, and mineral deposits were stained by Alizarin Red. It is evident that 19B8, 26E6, and 91E2 stimulate the formation of bonelike mineral deposits. Effects of IGF1 (10 ng/ml) and ipriflavone (10 μM) are shown as a positive control. *B*, RT-PCR analysis of osteocalcin. MC3T3-E1 cells were treated with chemicals for 3 days, and total RNA was isolated for RT-PCR analysis.

increased induction of osteocalcin after 3 days of incubation (Fig. 4*B*). These compounds may serve as a small molecule tool for the mechanistic analysis of osteogenesis, and such studies could lead to the development of pharmaceuticals for osteoporosis, one of the most underdiagnosed and undertreated disorders in medicine.

Suppressors of IGF-activated Cancer Cells—We next turned our attention to the 87 compounds that blocked the insulin-induced adipogenesis. Both insulin and IGFs stimulate oncogenic signaling pathways including those of Ras-MAPK and phosphatidylinositol 3-kinase-Akt, and overexpression of IGFs is often associated with cancer malignancy (25). Patients with IGF-overexpressing tumors tend to have severe hypoglycemia despite low levels of serum insulin (known as non-islet cell tumor hypoglycemia) (26), demonstrating a functional overlap between oncogenic IGFs and insulin *in vivo*. These considerations led to the hypothesis that the pool of the adipogenesis-blocking chemicals contains anti-cancer compounds that suppress the IGF-stimulated survival and proliferation of malignant tumor cells. We first examined whether the adipogenesis-blocking chemicals impair the viability of human hepatocellular carcinoma cells that overexpress IGF2, a member of IGFs that is often produced at high levels in liver tumors (27). We identified three chemically analogous compounds that killed IGF2-overexpressing hepatocellular carcinoma cells (Hep-G2) but had milder effects on the cell line with low levels

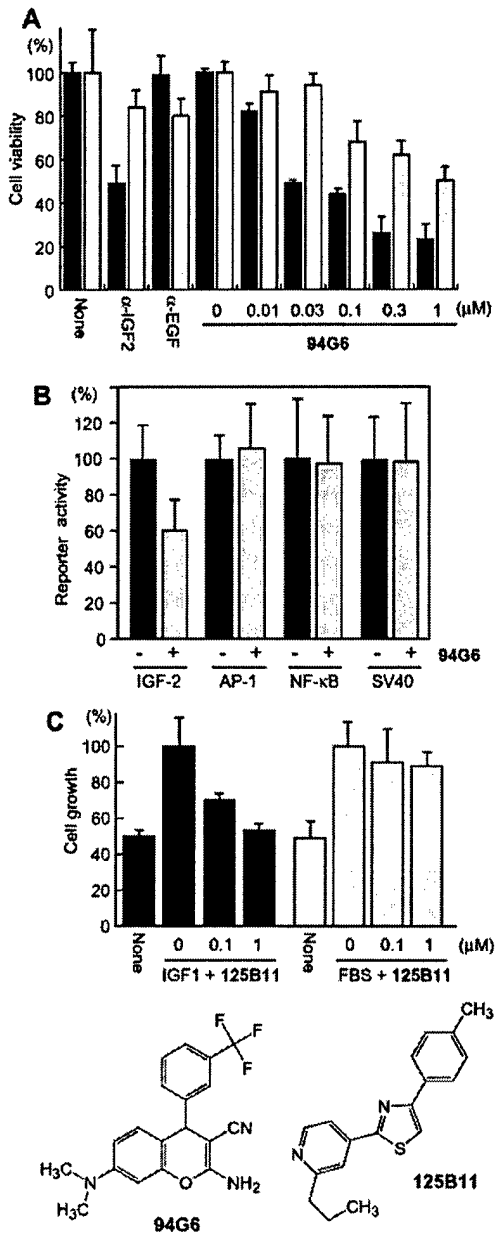

FIG. 5. **Discovery of anti-cancer compounds from the adipogenesis-blocking chemicals.** *A*, hepatocellular carcinoma cell lines, Hep-G2 (*black bars*) and SK-Hep-1 (*gray bars*), were treated with varied amounts of 94G6. 94G6 selectively impaired the viability of IGF2-overexpressing Hep-G2 but had much milder effects on SK-Hep-1 with low levels of IGF2. 94G6 was as selective as a neutralizing antibody against IGF2 (100 μg/ml). The cell viability was estimated by MTT assays in triplicate. *B*, specific inhibition of the IGF2 promoter by 94G6. Hepatocellular carcinoma cells were transiently transfected with a reporter construct in which a gene encoding SEAP is controlled by the IGF2 promoter, AP-1 sites, κB sites, or the SV40 promoter. The transfected cells were treated with 0.1 μM 94G6 for 8 h, and SEAP activity was measured through fluorescence change of a fluorogenic substrate. *C*, 125B11 inhibited the IGF1-induced growth but not the serum-induced growth. DU-145 cells were treated with varied amounts of 125B11 in the presence of IGF1 or 2% fetal bovine serum (*FBS*).

of IGF2 (SK-Hep-1) (28). Repeated experiments with three additional human hepatocellular carcinoma cell lines that we recently characterized[2] indicated that one of the three chemicals, 94G6, exhibited the highest cytotoxicity to IGF2-producing hepatocellular carcinoma cells with selectivity similar to that of a neutralizing antibody against IGF2 (Fig. 5*A*). This

benzochromene derivative killed the IGF2-producing cells at an IC_{50} of 29 nM but had ~33 times weaker effects on the hepatocellular carcinoma cells with low level of IGF2. Reporter gene transcription assays showed that 94G6 selectively inhibits the promoter of IGF2 in the hepatocellular carcinoma cells, suggesting that 94G6 blocks the autocrine loop of IGF2 (Fig. 5*B*). Although 94G6 may target multiple cellular events for causing cell death, the selective inhibition of the IGF2 autocrine loop provides a reasonable explanation for its inhibitory effects on adipogenesis and cancer cell survival.

Another type of IGF-associated tumors is prostate cancer, one of the most common malignant tumors in Western countries. Elevated levels of circulating IGF1 are strongly associated with the risk of developing prostate cancer, and modulation of IGF1 functions by small molecules is an attractive therapeutic approach when combined with androgen-targeting therapies (29). For a chemical screen, we used DU-145 androgen-independent prostate cancer cells whose growth can be stimulated by IGF1 by as much as 2% serum. The pool of the adipogenesis-blocking chemicals contained two analogous chemicals that specifically inhibited the IGF1-induced growth of DU-145 cells but had little effects on their serum-induced growth. One of them, 125B11, had the greatest differential activity in which the simple druglike thiazole derivative impaired the IGF1-induced growth at an IC_{50} of 0.1 μM but had little effects on the serum-dependent growth (Fig. 5*C*). IGF1-induced phosphorylation of Akt and MAPK in DU-145 cells was unaffected by 125B11, suggesting that 125B11 inhibits the cell-proliferative function of IGF1 in a way independent of the known IGF1-signaling pathway. Deregulation of the IGF axis is associated with the initiation and progression of many types of human carcinoma including breast (30) and colorectal cancers (31). A focused library of adipogenesis-blocking chemicals may serve as a source of anti-proliferative agents against the IGF-linked cancers.

DISCUSSION

Fat cell differentiation *per se* has no direct link to glucose uptake, cytokine inhibition, osteogenesis, and selective suppression of cancer cells. Nevertheless, our proof-of-principle study using a 10,000-compound library successfully identified non-cytotoxic bioactive compounds for these seemingly disparate pharmacological effects, just as genetics has identified non-lethal disease-linked genes by examining the eye morphology of fruit flies. We randomly picked up 70 compounds that had no detectable phenotypes in the adipogenesis profiling and assayed for their ability to modulate glucose uptake, cytokine production, IGF-selective cytotoxicity, and osteogenesis. As expected, no significant hits were found in each assay, indicating that the adipogenesis profiling with 3T3-L1 cells is a good filter at least for these pharmacological effects. A data base search revealed that one of the adipogenesis-enhancing chemicals has been patented as an inhibitor of neuropeptide Y, a proposed attenuator of insulin and leptin that stimulates appetite (32). Neuropeptide Y inhibitors are expected to treat feeding disorders and heart diseases (33). Adipogenesis profiling may find use in discovering chemicals with such biological effects. The insulin family of hormones is involved in many other conditions as observed in the complications of hyperinsulinism. The insulin-linked pharmacological effects including wound healing and anti-apoptosis (34) may be expected in adipogenesis-modulating compounds.

One potential drawback of our approach is that the bioactive molecules from the adipogenesis-based focused library may have side effects that are associated with adipogenesis. However, some degree of side effects are usually expected for any unoptimized molecules, and classical medicinal chemistry approaches have been taken for reducing the unwanted side effects. The high sensitivity of the morphological transformation of 3T3-L1 cells also suggests that the adipogenesis-modulating effects of chemicals may not necessarily be reproduced in human. For instance, non-steroidal anti-inflammatory drugs and phosphodiesterase inhibitors such as aspirin and caffeine are known to enhance adipogenesis of 3T3-L1 cells but have no significant effects on fat accumulation in human. The adipogenesis profiling is perhaps a good filter for lead-like bioactive molecules that can be used for further biological, chemical genetic, and medicinal chemical studies.

Adipogenesis-based profiling of more chemical compounds including clinically proven drugs would catalog the biological activities of small organic molecules and help to design a focused chemical library that is small enough to be screened with unique low throughput assays yet generates drug seeds for a broad range of disease conditions. Systematic chemical genetic studies on morphological changes of cells could provide small molecule tools for biological studies of human diseases as found in the role of developmental biology in the analysis of disease-linked genes.

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